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Biotransformation of BDE-47 to Potentially Toxic Metabolites Is Predominantly Mediated by Human CYP2B6

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Abbreviations

BDE	bromodiphenyl ether
BDE-17	2,2',4-tribromodiphenyl ether
BDE-28	2,4,4'-tribromodiphenyl ether
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether
BDE-99	2,2',4,4',5-pentabromodiphenyl ether
BDE-209	2,2′,3,3′,4,4′,5,5′,6,6′-deca-bromodiphenyl ether
BSA	bovine serum albumin
^{13}C-labeled 6-OH-BDE-47	^{13}C-labeled 6-hydroxy-2,2′,4,4′-tetrabromo diphenyl ether
CYPs	cytochrome P450s
DMSO	dimethyl sulfoxide
EI	electron impact
GC-MS	gas chromatography with mass spectrometry

GC-MS/MS	gas chromatography with tandem mass spectrometry
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLMs	human liver microsomes
K_m	Michaelis rate constant
MeO-BDE	methoxylated bromo diphenyl ether
MLODs	Method limits of detection
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
nmol	nanomoles
OH-PBDEs	hydroxylated PBDEs
3-OH BDE-47	3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether
4-OH-BDE-42	4-hydroxy-2,2′,3,4′- tetrabromodiphenyl ether
4′-OH-BDE-49	4′-hydroxy-2,2′,4,5′- tetrabromodiphenyl ether
5-OH-BDE-47	5- hydroxy-2,2′,4,4′-tetrabromodiphenyl ether
6-OH-BDE-47	6-hydroxy 2,2′,4,4′-tetrabromodiphenyl ether
PBDE	polybrominated diphenyl ether
pmol	picomoles
PTV	programmable temperature vaporizing
SRM	selective reaction monitoring
TBG	thyroxine-binding globulin
TTR	transthyretin
µM	micromolar
V_{max}	Maximum rate

Abstract

Background: Previous studies have indicated that cytochrome P450s (CYPs) are involved in the metabolism of polybrominated diphenyl ether (PBDE) flame retardants in humans resulting in the formation of hydroxylated PBDEs (OH-PBDEs) that are potentially more toxic than the parent PBDEs. However, specific enzymes responsible for the formation of OH-PBDEs are not known.

Objectives: The purpose of this study was to characterize the *in vitro* metabolism of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) by human liver microsomes (HLM) and recombinant human cytochrome P450s, and to identify the CYP(s) that are active in the oxidative metabolism of BDE-47.

Methods: Recombinant human CYPs (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4) were incubated with BDE-47 (20 μM). Metabolites were measured and characterized using gas chromatography with tandem mass spectrometry (GC-MS/MS). For kinetic studies, CYP2B6 and pooled human liver microsomes (HLMs) were incubated with BDE-47 (0-60 μM).

Results: CYP2B6 was the predominant CYP capable of forming six OH-BDEs, including 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4-OH-BDE-42, 4′-OH-BDE-49 and a metabolite tentatively identified as 2′-OH-BDE-66. Based on full-scan GC-MS analysis we hypothesized the formation of a di-OH-tetra-BDE and di-OH-tetrabrominated dioxin metabolites. Kinetic studies of BDE-47 metabolism by CYP2B6 and pooled HLMs found K_m values ranging from 3.8-6.4 μM and 7.0-11.4 μM, respectively, indicating the high affinity towards the formation of OH-BDEs.

Conclusion: Our findings support the predominant role of CYP2B6 in the metabolism of BDE-47 to potentially toxic metabolites, including a hypothesized di-OH-tetrabrominated dioxin

metabolite. These results will assist future epidemiological studies investigating the potential of PBDEs and their metabolites to produce neurobehavioral / neurodevelopmental disorders.

Introduction

During the past decade both animal and human studies have supported an association between polybrominated diphenyl ether (PBDE) flame retardants and neurobehavioral / neurodevelopmental disorders, particularly following *in utero* and postnatal exposure (Darnerud et al. 2001; Eriksson et al. 2001; Costa and Giordano 2007; Suvorov et al. 2009; Roze et al. 2009; Herbstman et al. 2010). While three mixtures of PBDEs were produced globally (penta-BDE, octa-BDE, and deca-BDE), the lower brominated mixtures contain the more bioaccumulative and persistent congeners, with 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) representing the most abundant PBDE detected in human serum (Birnbaum and Staskal 2004). The use and production of penta-BDE and octa-BDE have been banned in Europe since 2004, and were also voluntarily phased out in the United States at the end of 2004. However, human and environmental levels of penta-BDEs are about 10-fold higher in North America compared with Europe and Asia, while 2,2′,3,3′,4,4′,5,5′,6,6′-deca-bromodiphenyl ether (BDE-209) levels are higher in Asia (Frederiksen et al. 2009; Law et al. 2008).

While the ingestion of contaminated meat, fish and dairy products is a known source of human exposure, the wide-spread use of PBDEs in electrical appliances, plastics, televisions, drapes and upholstered furniture for households has contributed to human exposure through inhalation and ingestion of household air and dust (Allen et al. 2007; Johnson et al. 2010). In contrast to other persistent organic pollutants, the highest body burdens of PBDEs are found in infants and toddlers, due primarily to exposure to maternal milk and house dust containing PBDEs (Costa et al. 2008; Fischer et al. 2006; Rose et al. 2010; Schecter et al. 2004). These findings are of concern since a study of New York City children aged 72 months and younger found that lower scores on tests of cognitive, behavioral, and physical development were associated with higher

levels of PBDEs in cord blood, which was used as a measure of perinatal exposure (Herbstman et al. 2010). In 6 year old Dutch children, maternal PBDE levels in the 35th week of pregnancy were correlated with impairments in fine psychomotor abilities and attention, but also correlated with better coordination, better visual perception, and better behavior (Roze et al. 2009). In addition, an inverse association between PBDEs and serum thyroid-stimulating hormone (TSH) concentrations has been reported in pregnant women. Low TSH levels may result in maternal subclinical hyperthyroidism, which in turn has been associated with adverse pregnancy outcomes and potential effects on fetal and child development (Chevrier et al. 2010). Recently, hydroxylated metabolites of PBDEs (OH-PBDEs) have been found to accumulate in human serum at levels similar to, and in some cases greater than, that of the parent PBDEs (Qiu et al. 2009; Athanasiadou et al. 2008). The significance of this finding is heightened by mechanistic studies showing that mono-hydroxylated metabolites of BDE-47 are more potent than the parent BDE-47 in disrupting Ca^{2+} homeostasis, modulating GABA and alpha4beta2 nicotinic acetylcholine (nACh) receptor function, altering spontaneous activity and cell viability in cultured cortical neurons, and competing with thyroxine (T4) for binding to human transthyretin (TTR) (Kim et al. 2011; Dingemans et al. 2008; Dingemans et al. 2010a; Dingemans et al. 2010b; Dingemans et al. 2011; Hendriks et al. 2010; Hamers et al. 2008). Together, these and other studies suggest that bioactivation by oxidative metabolism adds considerably to the neurotoxic potential of PBDEs. Thus, there is a critical need to further our understanding of the factors affecting PBDE metabolism and accumulation of metabolites in humans.

Recently Stapleton et al (Stapleton et al. 2009) examined metabolism of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) and BDE-209 in fresh human hepatocytes. The study reported the formation of monohydroxylated pentabrominated diphenyl ether metabolites for

BDE 99, while BDE-209 appeared to be not metabolized (Stapleton et al. 2009). Moreover, we have previously demonstrated that human liver microsomes were capable of metabolizing BDE-47 and BDE-99 to OH-BDEs (Lupton et al. 2009; Lupton et al. 2010). The oxidative metabolism of BDE-47 and BDE 99 was also studied in rat hepatic microsomes, and OH-PBDE metabolites were identified for both BDE congeners (Harmers et al. 2008; Erratico et al. 2011).

The purpose of this study was to conduct a qualitative and quantitative characterization of the *in vitro* metabolism of BDE-47 utilizing human liver microsomes and recombinant human cytochrome P-450s (CYPs), and to identify the CYP(s) that are most active in the oxidative metabolism of this persistent BDE congener.

Materials and Methods

Standards and Reagents

Pooled human liver microsomes (HLMs) from 50 donors were purchased from Xenotech (Lenexa, KS), Baculovirus-insect cell microsomes (BD Supersomes) containing individually expressed human CYPs (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4) co-expressed with human CYP reductase and cytochrome b_5, with characterized CYP protein levels and activities, were obtained from BD Biosciences (San Jose, CA).

Commercially available standards of 2,2',4-tribromodiphenyl ether (BDE-17), 2,4,4'-tribromodiphenyl ether (BDE-28), BDE-47, and BDE 99 used as internal standard, were purchased from Accustandard, Inc. (New Haven, CT). A reference standard of ^{13}C-labeled 6-hydroxy-2,2′,4,4′-tetrabromo diphenyl ether (^{13}C-labeled 6-OH-BDE-47), used as surrogate, was

obtained from Wellington Laboratories (Guelph, ON, Canada). Neat BDE-47 was purchased from Chem Service (West Chester, PA).

The methoxylated (MeO) analog standards of 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (3-OH BDE-47), 5-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (5-OH-BDE-47), 6-hydroxy 2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47), 4′-hydroxy-2,2′,4,5′- tetrabromodiphenyl ether (4′-OH-BDE-49) and 4-hydroxy-2,2′,3,4′- tetrabromodiphenyl ether (4-OH-BDE-42) (Wellington Laboratories) were all gifted from Dr. Mehran Alaee (Environment Canada). Trimethylsilyl diazomethane, used for methylation of OH-PBDEs, was obtained from Sigma-Aldrich (St. Louis, MO).

CYP Specific Metabolism of BDE-47. Recombinant human CYPs including CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 (0.5 mg; 38.5 pmol CYP2B6 to 256 pmol CYP2C9) were individually incubated with 20 μM BDE-47 (which represented saturating conditions, see Supplemental Material, Figure S1 and S2) (DMSO vehicle, 0.5% v/v) in 1-mL of buffer consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 6 mM magnesium chloride, 0.8 mg/mL bovine serum albumin (BSA) as a BDE carrier, and 1 mM NADPH at pH 7.4. The assay was initiated with NADPH and incubated for 2h at 37 ˚C to assess the potential of human CYPs to biotransform BDE-47. Samples were frozen and stored at -20 ºC until analysis. Control samples consisting of BDE-47 incubations in the absence of microsomal protein (to determine any abiotic transformation of BDE-47), human liver microsomes incubated in the absence of BDE-47 (to determine any background levels of OH-BDEs and MeO-BDEs), and recombinant CYPs incubated in the absence of BDE-47 (to monitor contamination) were included in the study. Control samples were prepared in duplicate.

Kinetics for Metabolism of BDE-47. For kinetic studies, recombinant human CYP2B6 (40 pmol) and pooled human liver microsomes (HLMs; 0.298 mg, 140 pmol total CYP) were incubated with BDE-47 (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 40 and 60 μM) for 60 min using the incubation conditions described above. Incubation time was previously determined to ensure linear formation of metabolites over time. Experiments were done in triplicate. The kinetic values, V_{max} and $K_{m,}$ were determined by nonlinear regression analysis (Origin (OriginLab Corporation, V8)) of hyperbolic plots (i.e., velocity vs. substrate concentration [S]) obeying Michaelis-Menten kinetics.

BDE-47 and metabolites extraction. After incubation, each of the 1-mL sample was spiked with 0.2 μg of ^{13}C-BDE-47 and 0.2 μg of ^{13}C-6-OH-BDE-47 which served as surrogates. Samples were extracted with 3-mL 75/25 (v/v) solution of hexane/ dichloromethane after adding 0.5-mL 6M HCl for protein denaturation. The mixtures were vortexed and centrifuged, and then the organic layer was removed from each sample. A second addition of 3-mL 50/50 (v/v) solution of hexane/dichloromethane was added, for extraction. The organic layer was removed and combined with the previous organic layer.

The extracts were evaporated to dryness under a stream of nitrogen and reconstituted in 0.4 mL 50/50 (v/v) hexane/dichloromethane. Then, 0.2 mL of each extract was collected and evaporated to dryness and reconstituted in 0.2 mL of toluene for analysis of BDE-47 by gas chromatography with tandem mass spectrometry (GC-MS/MS). Full scan GC-MS analysis was performed in order to check for the possible formation of debrominated metabolites. The other 0.2 mL aliquot was used for the analysis of OH-BDE-47 metabolites by first derivatizing the sample with trimethylsilyl diazomethane. The reaction was performed for over 18 h in a mixture containing 0.2 mL of extract, 0.1 mL of methanol, 0.05 mL of hexane, and 0.25 mL of trimethylsilyl

diazomethane. After derivatization, samples were evaporated to dryness using a slow stream of nitrogen, and reconstituted in 3 mL hexane. A 3-mL aliquot of acetic acid (glacial acetic acid, Sigma Aldrich, USA) was added to each sample to remove excess derivatizing agent. The mixture was then vortexed and centrifuged to separate the organic layer. The latter was then transferred into a separate test tube, evaporated to dryness under a stream of nitrogen, and then reconstituted with 0.1 mL toluene for analyses. Samples were analyzed under full scan GC-MS for the identification of the metabolites, and then by selective reaction monitoring (SRM) GC-MS/MS for the quantification of the derivatized OH-PBDE metabolites.

GC-MS and GC-MS/MS analysis. The underivatized and derivatized extracts were analyzed using a gas chromatograph (Trace GC Ultra, Thermo Scientific) coupled to a triple quadrupole mass spectrometer (TSQ Quantum XLS, Thermo Scientific) and equipped with a programmable temperature vaporizing (PTV) injection system. All samples were monitored under electron impact (EI) ionization. Separation was performed on a Zebron Inferno™ ZB-5HT column (15 m x 0.25 mm id x 0.10 µm film thickness) (Phenomenex, Torrance, CA). The PTV inlet had an initial temperature of 80 ºC, and was increased at a rate of 2.5 ºC/sec for 1 min. The carrier gas (Helium) was 1.2 mL/min and injection was set in splitless mode. For the analysis of PBDEs the oven temperature was programmed to start at 100 °C (held for 2 min), then increased to 250 °C at 25 °C min^{-1}, and finally to 300 °C at 20 °C min^{-1}. The oven temperature was held at 300 °C for 5 min before it was returned to the initial temperature. For the analysis of metabolites, the oven temperature started at 100 °C (held for 2 min), then increased to 250 °C at 10 °C min^{-1}, and finally to 300 °C at 15 °C min^{-1}. The oven temperature was held at 300 °C for 5 min, then returned back to the initial temperature.

The GC-MS was initially operated under full scan mode (scanning from *m/z* 100 to 650) to confirm the fragment ions (*m/z*) corresponding to BDE-47, and to determine molecular ions [M+] of the PBDE metabolites formed. Once the *m/z* values have been identified, GC-MS/MS analysis was performed under SRM mode (*m/z* 485→326 and 485→328 for BDE-47; *m/z* 516→356 and 516→358 for the OH-PBDE metabolites).

Quality control. Regarding GC-MS/MS analysis**,** extraction recoveries were determined from samples spiked with BDE-47 (9.7 µg) containing heat-inactivated microsomes. Quantification was done based on a standard calibration curve, using ^{13}C-BDE-47 (0.2 µg) as surrogate (added prior to extraction) to correct for recoveries. Furthermore, BDE-99 (0.2 µg) was added as internal standard (added after extraction and clean-up, just prior to GC-MS analysis) to check for instrument performance. Recoveries were between 100-110%. Samples were analyzed in duplicate.

For OH-PBDE metabolites, ^{13}C-6-OH-BDE-47 was used to determine the overall derivatization and extraction recoveries. A 0.2 µg aliquot of ^{13}C-labeled 6-OH-BDE-47 was spiked into the sample after incubation, and then extracted and derivatized as previously described. The recovery of ^{13}C-6-OH-BDE-47 was estimated through quantification of ^{13}C-6-OH-BDE-47 by an external standard calibration curve (6.25, 12.5, 25, 50, 100, 200 ng/mL) of 6-MeO-BDE-47. As described above, BDE-99 (5 ng) was used as internal standard. The overall averaged spike recoveries were greater than 60%.

Quantification of the other OH-PBDE metabolites in the samples was achieved based on external standard calibration curves (6.25, 12.5, 25, 50, 100, 200 ng/mL) using commercially available MeO-PBDE standards containing 3-MeO-BDE-47, 5-MeO-BDE-47 and 6-MeO-BDE-47, 4′-

MeO-BDE-49, and 4-MeO-BDE-42. Again, BDE-99 (5 ng) was used as internal standard for these samples.

All the standards used for calibration curve were prepared in toluene.

Method limits of detection (MLODs) and method limits of quantification (MLOQs) of the GC/MS/MS were determined for the three OH-BDEs included in the kinetics study (3-OH-BDE, 5-OH-BDE, and 6-OH-BDE). MLOD and MLOQ are defined as the minimum amount of analyte that produces a peak with a signal-to-noise ratio equal to 3 and 10, respectively. Following the approach described by Harris (Harris 2011), the MLOD is about 2 pg, and the MLOQ is about 7 pg, for the three major OH-BDEs detected in this study.

Results.

CYP-specific metabolism of BDE-47

BDE-47 (20 μM) was incubated with eleven individual recombinant human CYPs (see Table 1) for 120 min to assess the relative formation of monohydroxylated metabolites under the conditions described above. In Table 1, the relative amount of metabolite formed by each individual CYP tested are expressed in terms of the chromatographic peak area per nmol of each respective CYP and expressed relative to the area formed by CYP2B6 (which was assigned as 100%). It is clear that CYP2B6 is the predominant CYP that is capable of metabolizing BDE-47 to the OH-BDEs identified in this study. Other CYPs that showed some activity towards PBDE oxidation, albeit at a much lesser extent, are CYP 2C19, and CYP3A4.

Identification of metabolites. Figure 1 shows the structures of the eight hydroxylated BDE-47 metabolites formed by recombinant human CYP2B6 and detected as methyl derivatives in the sample extracts. These metabolites were detected under full-scan GC/MS, and were tentatively assigned their structures based on the MS isotopic signature of bromine. Later, the identities of five of these metabolites were confirmed by comparison of their retention times and full mass spectra with authentic methoxylated BDE standards (3-MeO-BDE-47, 5-MeO-BDE-47 and 6-MeO-BDE-47, 4′-MeO-BDE-49 and 4-OH-BDE-42). Figure 2 shows a sample GC-MS/MS chromatogram in SRM mode of the derivatized OH-PBDEs formed during incubation of recombinant human CYP2B6 with BDE-47.

Analysis of metabolites formed by recombinant human CYP2B6-mediated oxidation of BDE-47 using full scan GC-MS suggested the possible formation of two other metabolites: di-OH-tetra-BDE (retention time, Rt = 15.01; m/z= 545.75) which co-eluted with 5-OH-BDE-47 (Figure 2c), and di-OH-tetrabrominated dioxin (Rt = 14.27; m/z= 558.75) which co-eluted with a monohydroxylated metabolite, tentatively identified as 2′-OH-BDE-66 (Figure 2b). The di-OH-tetra-BDE was also previously reported as one of the BDE-47 metabolites formed in HLM (Lupton et al. 2010). On the other hand, di-OH-tetrabrominated dioxin has not been reported previously.

Unfortunately, no commercially standards were available for 2′-OH-BDE-66 as well as for di-OH-tetrabrominated dioxin and di-OH-tetra-BDE so their identities cannot be confirmed. Structural analysis by nuclear magnetic resonance (NMR) was not possible due to the limited amount formed. However, the formation of 2′-OH-BDE-66 can be reasonably hypothesized, based on the full mass spectra of the derivatized metabolite (Figure 2b), and from previous studies reporting the presence of this PBDE metabolite in human blood samples (Athanasiadou

et al. 2008; Qiu et al. 2009) and in mice (Qiu et al. 2007; Hamers et al. 2008). Our hypothesis that 2′-OH-BDE-66 was formed, where the presence of OH is in the ortho position to the diphenyl ether bond, was supported by the full-scan mass spectra showing significant abundance of m/z 420 (loss of $BrCH_3$). In the systematic study by Athanasiadou et al. (2006), where they characterized the fragmentation pattern of twenty-six methoxylated PBDEs by GC/MS, it was shown that ortho-MeO-PBDEs all showed a loss of $BrCH_3$ (M-94) in the EI mode, which was absent or hardly observable for *meta*-MeO-PBDEs and *para*-MeO-PBDEs. Therefore, the presence of m/z 420 (M-94) in Figure 2b, suggests that the derivatized OH-BDE formed an ortho-MeO-BDE corresponding to the proposed 2′-OH-BDE-66. Nevertheless, this proposed structure needs confirmation using authentic standards when available.

In control samples, OH-PBDEs were not detected in microsomal incubations in the absence of BDE-47. Underivatized samples, prepared for the analysis and quantification of BDE 47, were also analyzed for possible MeO-PBDE metabolites or contamination. The analysis showed that MeO-PBDEs were not present in the underivatized samples (data not shown). Possible metabolites formed by reductive debromination were observed in the analysis of underivatized samples. However, reductive debromination was also observed in the control samples involving BDE-47 incubated in the absence of microsomal proteins. All reductive debromination resulted in the formation of BDE-17 and BDE-28, which were identified based on their retention times and full mass spectra matching those of authentic reference compounds. Due to its presence within the control samples, debromination was attributed to abiotic processes.

Kinetic study

The formation of 3-OH-BDE-47, 5-OH-BDE-47, and 6-OH-BDE-47 (which were the three monohydroxylated BDE-47 metabolites and were, detectable at lower (0.1 μM) BDE-47 incubation levels) were evaluated over a range of BDE-47 concentrations (0.1 to 60 μM) at an incubation time of 60 min using recombinant human CYP2B6 and pooled HLM. Table 2 shows the kinetic parameters (Km and Vmax) obtained from Michaelis-Menten plots, based on the rate of OH-PBDE metabolite formation during incubation of BDE-47 with CYP2B6 and HLM. Lower Km values were found (6.4, 3.8 and 4.2 μM for 3-OH-BDE-47, 5-OH-BDE-47 and 6-OH-BDE-47, respectively) during the incubation of BDE-47 with CYP2B6, relative to those observed with HLM (11.4, 7.3, and 7.0 μM for 3-OH-BDE-47, 5-OH-BDE-47 and 6-OH-BDE-47, respectively) (see Supplemental Material, Figure S1 and S2). The 5-OH-BDE-47 and 6-OH-BDE-47 showed the lowest Km values and were the major metabolites produced by BDE-47 in both CYP2B6 (Vmax=948 pmol/min/nmol P450 and Vmax=202 pmol/min/nmol P450, respectively) and HLM (Vmax=227 pmol/ min/nmol P450 and Vmax=39.3 pmol/ min/nmol P450, respectively) (Table 2).

Discussion

Out of the eleven recombinant cytochrome P-450s that were screened, CYP2B6 was the predominant enzyme capable of transforming BDE-47 into 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4-OH-BDE-42, 4'-OH-BDE-49 and a metabolite tentatively identified as 2'-OH-BDE-66 (Table 1). As illustrated in Figure 1, the formation of one ortho- hydroxylated (2'-OH-BDE-66) and two para- hydroxylated (4-OH-BDE-42 and 4'-OH'BDE-49) metabolites are

proposed to result from an NIH-shift of a bromine atom in concert with formation of an arene oxide intermediate (Boyd and Sharma 1996). The results further suggested that CYP2B6 catalyzed the formation of two additional metabolites (Figure 1). One metabolite, dihydroxylated tetra-BDE (di-OH-tetra-BDE), has been previously reported in our earlier publication (Lupton et al. 2010), but this present study is the first to suggest the di-OH-tetrabrominated dioxin formation by CYPs. However, the identity of these metabolites cannot be confirmed due to the lack of commercially available standards. Moreover, the specific location of bromination and hydroxylation in dihydroxylated tetra-BDE and in di-OH-tetrabrominated dioxin was not determined because of the limited amount of metabolite formed to allow further characterization. However, mass spectral data confirmed that both dihydroxylated metabolites contain four bromines characteristic of the bromine isotopes.

With the exception of 2'-OH-BDE-66, all of the mono-OH BDE metabolites detected in this study (Table 3) have been previously observed in human blood samples (Qiu et al. 2009; Athanasiadou et al. 2008), suggesting that the pathway for *in vitro* metabolism of BDE-47 by HLM reflects the *in vivo* biotransformation seen in humans well.

However, it is important to point that OH-BDEs detected in human blood could also come from the diet and natural sources. In fact, OH-BDEs and MeO-BDEs, have been identified as natural products produced by marine invertebrates and are present throughout the marine food web (Wiseman et al. 2011). The OH-PBDEs also accumulate in the abiotic environment including surface water, rainfall, snow, and wastewater and sediments as possible products formed via reaction of PBDEs with atmospheric OH radicals (Ueno et al. 2008). This means that OH-PBDEs in abiotic samples could be an important source for organisms and therefore influencing their concentrations in biota and humans through trophic transfer.

As presented in Table 1, CYP2C19 and CYP3A4 appear to be very minor contributors in the formation of OH-BDE metabolites from BDE-47. On the other hand, CYP2B6 plays a major role in the formation of OH-BDE metabolites. Kinetic studies of BDE-47 metabolism by CYP2B6 and HLM showed that the formation of 3-OH-BDE-47, 5-OH-BDE-47 and 6-OH-BDE-47 occurs at low BDE-47 concentrations, with apparent Km values in the range of 3.8-6.4 μM and 7.0-11.4 μM, for CYP2B6 and HLM, respectively. The relatively low Km values suggest that CYP2B6 has high affinity for BDE-47, although low μM levels of BDE-47 are in excess of those found in human tissues.

Recent mechanistic studies have shown that some OH-BDEs are more potent than parent compounds and may contribute substantially to neurodevelopmental disorders by direct neurotoxicity, or indirectly through altered thyroid disruption (Kim et al. 2011; Dingemans et al. 2008; Dingemans et al. 2010a; Dingemans et al. 2010b; Dingemans et al. 2011; Hendriks et al. 2010; Harmers et al. 2008). In contrast to BDE-47, which was without activity, the 6-OH-BDE-47 and 4'-OH-BDE-49 metabolites have recently been reported to be potent modulators of ryanodine receptors type 1 and 2, which regulate essential aspects of Ca^{2+} signaling (Kim et al. 2011; Pessah et al. 2010). It was shown that 6-OH-BDE-47 is more potent at mobilizing Ca^{2+} from endoplasmic and mitochondrial stores than the parent compound (Dingemans et al. 2008; Digemans et al. 2010a; Digemans et al. 2010b). The 6-OH-BDE-47 was also reported to modulate human GABAA and alpha4beta2 nicotinic acetylcholine (nACh) receptors (Hendriks et al. 2010). These results are of added importance because CYP2B6 is present in most regions of the human brain (Miksys et al. 2003; Miksys and Tyndale 2004) and the local production of active metabolites may contribute directly to localized alterations in Ca^{2+} homeostasis, leading to altered brain development and neurodevelopmental disorders.

Six mono-hydroxylated metabolites of BDE-47 have also been shown to have thyroxine transport disrupting activity (Marchesini et al. 2008). In fact, OH-PBDEs showed binding potency for thyroxine-binding globulin (TBG) and transthyretin (TTR) which are the two major thryroxine transport proteins in human plasma, each carrying 74% and 20% of total T4 (Cao et al. 2010; Marchesini et al. 2008). In particular, it has been found that OH-PBDEs were moderate to strong binders to TTR and slight to moderate binders to TBG (Marchesini et al. 2008). Thus OH-PBDEs are able to compete with natural hormone T4 in binding either TTR or TBG (Marchesini et al. 2008).

The 3-meta-OH group with two adjacent halogens present in 3-OH-BDE-47 has been shown to provide the optimum structure for binding to TTR (Marchesini et al. 2008). The affinity ranking for TTR has been reported to be 3-OH-BDE-47 > 5-OH-BDE-47 > 6OH-BDE-47 > 4'-OH-BDE-49, while the TBG affinity ranking was reported to be 6-OH-BDE-47 > 3-OH-BDE-47 > 5-OH-BDE-47 > 4'-OH-BDE-49 (Marchesini et al. 2008). Thus, the formation of mono-OH-BDEs in humans is an important issue if we consider that TTR is critical for maternal to fetal transport of thyroid hormones and for delivery of T4 across the brain barrier (Schreiber 2002), while TBG has been linked to facilitate the iodine supply to the fetus that initially has no iodine reserve (Schussler 2000).

In addition to mediating BDE-47 metabolism, CYP2B6, is known to exhibit up to 100-fold inter-individual variability in hepatic protein expression, due to regulatory phenomena and common genetic polymorphisms (Lang et al. 2001; Lang et al. 2004; Zanger et al. 2007; Watanabe et al. 2010). Thus, in addition to variable exposures to PBDEs, genetic variability in the CYP-specific metabolism of PBDEs may contribute to inter-individual variability in the body burden of PBDEs and the formation of toxic metabolites.

Conclusion

CYP2B6 was identified as the major enzyme involved in metabolism of BDE-47. Five of the mono-OH-BDEs metabolites formed were identified based on comparison of their mass spectral fragmentation and GC-MS-MS retention times with commercially available standards. A sixth mono-OH-BDE metabolite was detected and tentatively identified as 2′-OH-BDE-66. However, its identity cannot be confirmed because of the lack of commercially available standard. In addition a di-OH-tetra-BDE and a di-OH-tetrabrominated dioxin were tentatively identified based on their mass spectral data; however, their identities can only be hypothesized and can not confirmed at this time due to the lack of commercially available standards. These results will ultimately better inform future mechanistic and epidemiological studies investigating the potential of PBDEs and their metabolites to produce neurobehavioral / neurodevelopmental disorders.

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Table 1. Screening to Assess CYP-Specific Metabolism of BDE-47.

Incubation with human CYPs*	**3-OH-BDE-47** Peak area/nmolCYP(10^6) (% relative to CYP 2B6)	**5-OH-BDE-47** Peak area/nmolCYP (10^6) (% relative to CYP 2B6)	**6-OH-BDE-47** Peak area/nmolCYP (10^6) (% relative to CYP 2B6)	**4'-OH-BDE-49** Peak area/nmolCYP (10^6) (% relative to CYP 2B6)	**4-OH-BDE-42** Peak area/nmolCYP (10^6) (% relative to CYP 2B6)	**2'-OH-BDE-66*** ** Peak area/nmolCYP (10^6) (% relative to CYP2B6)
1A1, 1A2, 1B1	nd**	nd	nd	nd	nd	nd
2A6	nd	nd	nd	nd	nd	nd
2B6	3.83 (100)	10.2 (100)	4.61 (100)	2.36 (100)	0.758 (100)	1.51 (100)
2C9	nd	nd	nd	nd	nd	nd
2C19	0.00805 (0.210)	nd	nd	0.00783 (0.332)	nd	nd
2E1	nd	nd	nd	nd	nd	nd
3A4	0.0507 (1.32)	0.0244 (0.239)	nd	nd	nd	nd
2D6	nd	nd	nd	nd	nd	nd
2C8	nd	nd	nd	nd	nd	nd

*Individual recombinant CYPs were incubated for 120 with 20μM BDE-47. Peak areas are calculated based on chromatographs as shown in Figure 2a and expressed per nmol of each CYP. Numbers inside the parenthesis, which are bolded, are the percentage of the metabolite peak produced by a particular CYP relative to the response for CYP2B6.

** nd=not detected; peak areas below 1000 were considered below the detection limit of the metabolites in GC/MS/MS

*** no commercially standard was available for 2′-OH-BDE-66 so it was tentatively identified and its identity cannot be confirmed.

Table 2. Comparison of Kinetic parameters for the metabolism of BDE-47 using recombinant human CYP2B6 and pooled human liver microsomes (HLM).

Metabolites	**Recombinant CYP2B6**		**Pooled HLM**		
	K_m (μM)	V_{max} (pmol/min/nmol P450)	K_m (μM)	V_{max} (pmol/min/nmol P450)	V_{max} (pmol/min/mg protein)
3-OH-BDE47	6.4 ± 1.2*	10.6 ± 0.6	11.4 ± 1.4	1.9 ± 0.1	0.91 ± 0.04
5-OH-BDE47	3.8 ± 0.9	948 ± 56	7.3 ± 1.3	227 ± 12	107 ± 6
6-OH-BDE47	4.2 ± 0.8	202 ± 10	7.0 ± 0.9	39.3 ± 1.5	18.5 ± 0.7

*Values represent the mean ± SEM of three experiments.

Table 3. Metabolites of BDE-47 previously found in humans.

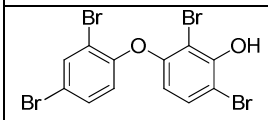
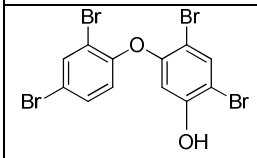
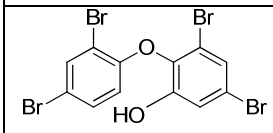
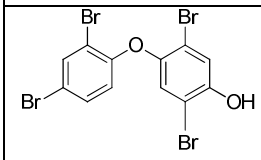
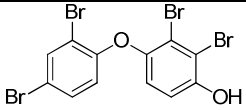
Metabolite	Detected in Human	Concentration	Sources
	Serum from Young Humans from Managua, Nicaragua	0.1-3 ng/g lw	Athanasiadou et al. 2008
	Fetal Blood from United States	1.6 ng/g lw	Qiu et al. 2009
3-OH-BDE-47	Maternal Blood from United States	0.1 ng/g lw	Qiu et al. 2009
	Fetal Blood from United States	28 ng/g lw	Qiu et al. 2009
5-OH-BDE-47	Maternal Blood from United States	1.6 ng/g lw	Qiu et al. 2009
	Serum of Young Humans from Managua, Nicaragua	0.1-6 ng/g lw	Athanasiadou et al. 2008
	Fetal Blood from United States	9.9 ng/g lw	Qiu et al. 2009
6-OH-BDE-47	Maternal Blood from United States	0.3 ng/g lw	Qiu et al. 2009
	Serum of Young Humans from Managua, Nicaragua	0.1-9 ng/g lw	Athanasiadou et al. 2008
4′-OH-BDE-49	Fetal Blood from United States	0.9 ng/g lw	Qiu et al. 2009
	Maternal Blood from United States	0.3 ng/g lw	Qiu et al. 2009
	Serum of Young Humans from Managua, Nicaragua	0.15-5 ng/g lw	Athanasiadou et al. 2008
4-OH-BDE-42			

Figure legends

Figure 1. Metabolic products of BDE-47 formed by CYP2B6. The structures of 2′-MeO-BDE-66, di-OH-BDE and di-OH-tetrabrominated dioxin metabolites are hypothesized based on mass spectra data.

Figure 2. **A)** Chromatogram of the BDE-47 metabolites monitored in GC-MS/MS in selective reaction monitoring (SRM) mode. **B)** Mass spectrum in full scan monitoring of 2′-HO-BDE-66 (M) and di-OH-tetrabrominated dioxin (M'). **C)** Mass spectrum in full scan monitoring of 5-OH-BDE 47 (M) and di-OH-BDE (M'). Due to the lack of commercially available standards for 2′-OH-BDE-66, di-OH-tetrabrominated dioxin and di-OH-BDE, the identity of these metabolites cannot be confirmed.

Figure 1.

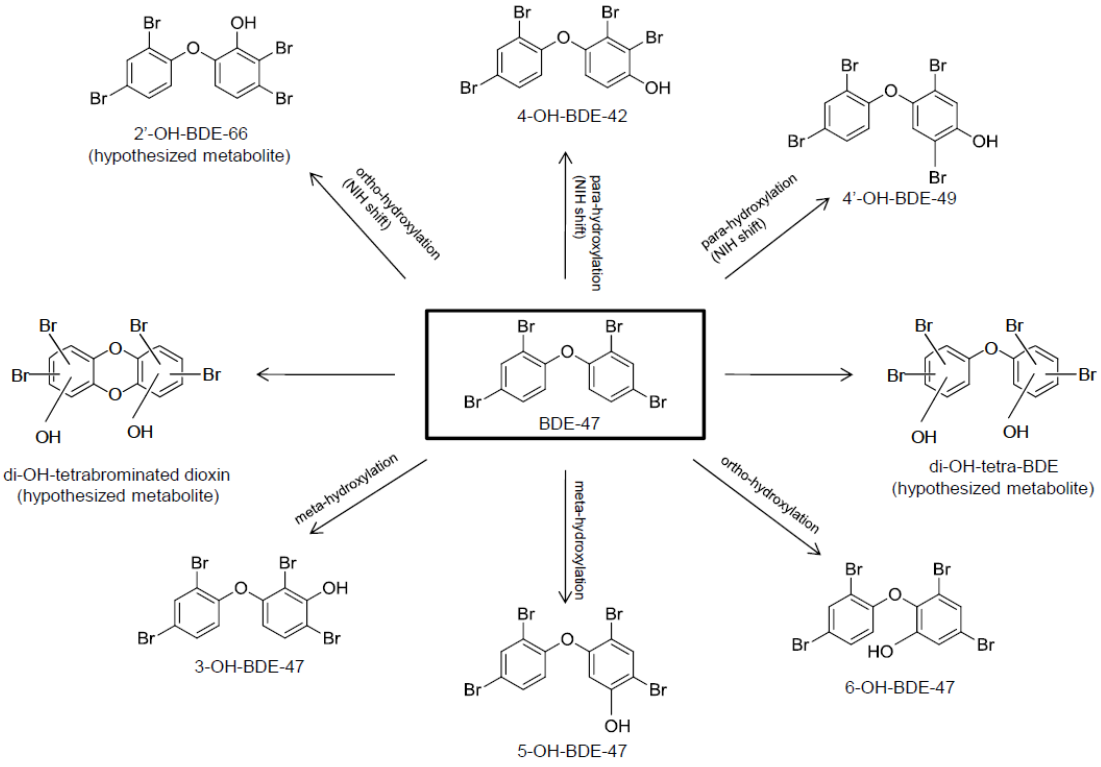
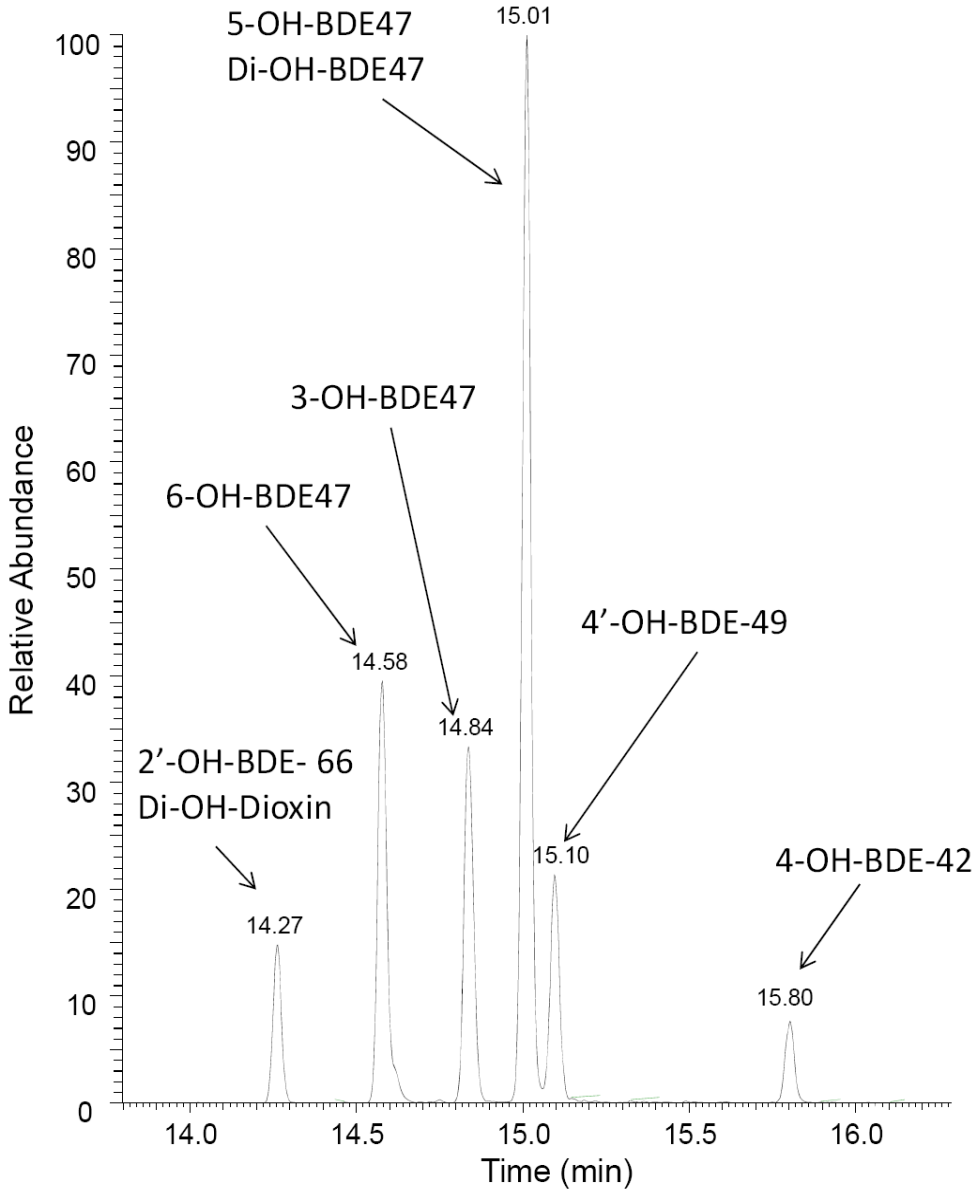
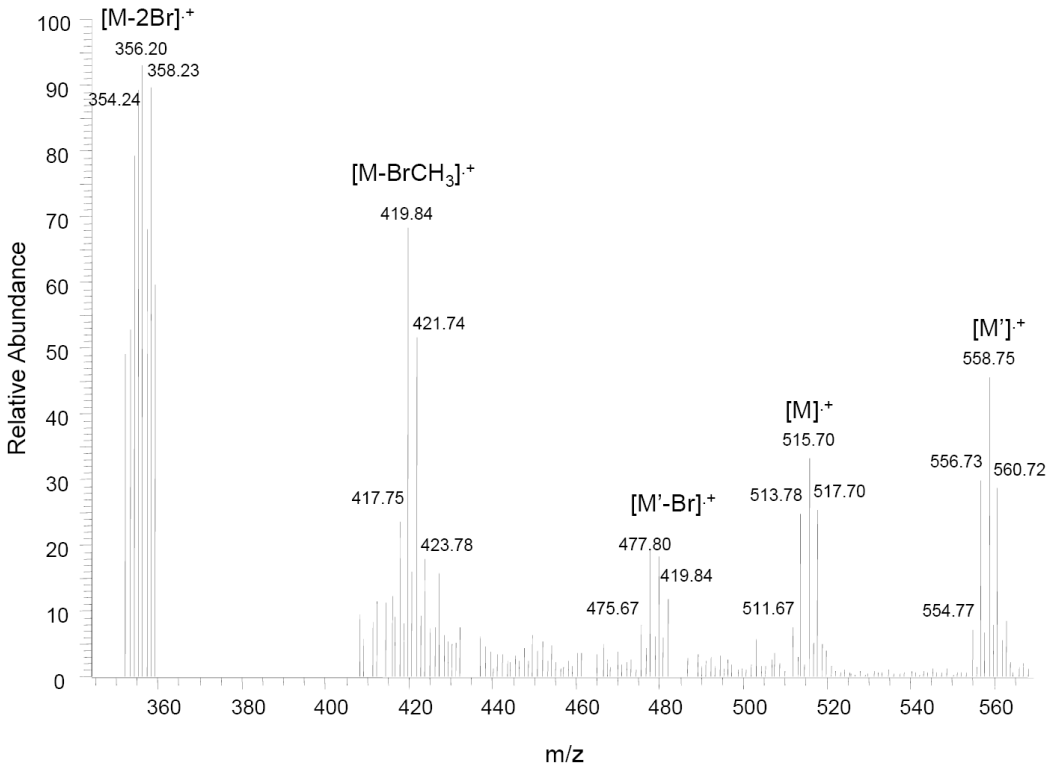

2'-OH-BDE-66
(hypothesized metabolite)
4-OH-BDE-42
4'-OH-BDE-49
ortho-hydroxylation
(NIH shift)
para-hydroxylation
(NIH shift)
para-hydroxylation
(NIH shift)
BDE-47
di-OH-tetrabrominated dioxin
(hypothesized metabolite)
di-OH-tetra-BDE
(hypothesized metabolite)
meta-hydroxylation
meta-hydroxylation
ortho-hydroxylation
3-OH-BDE-47
5-OH-BDE-47
6-OH-BDE-47

Figure 2

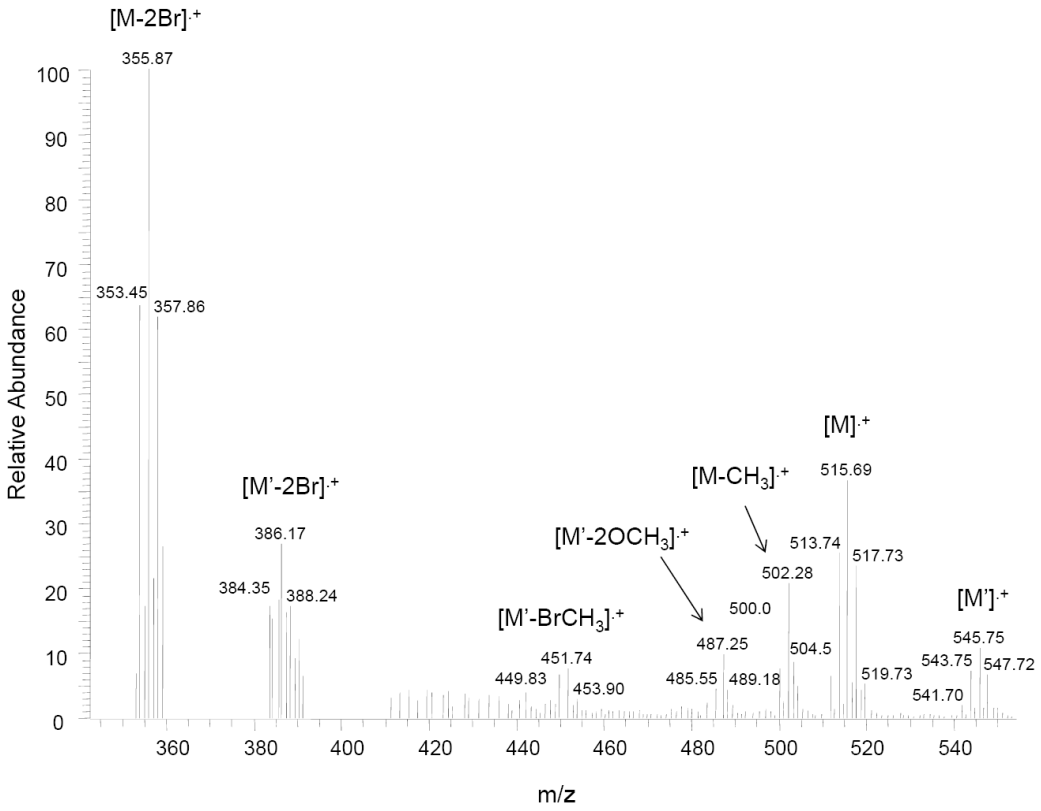
a)


5-OH-BDE47
Di-OH-BDE47
15.01
3-OH-BDE47
6-OH-BDE47
14.58
14.84
4'-OH-BDE-49
15.10
2'-OH-BDE- 66
Di-OH-Dioxin
14.27
4-OH-BDE-42
15.80
Relative Abundance
Time (min)
0
10
20
30
40
50
60
70
80
90
100
14.0
14.5
15.0
15.5
16.0

b)


[M-2Br]·+
356.20
358.23
354.24
[M-BrCH3]·+
419.84
421.74
417.75
423.78
[M'-Br]·+
477.80
419.84
475.67
[M]·+
515.70
513.78
517.70
511.67
[M']·+
558.75
556.73
560.72
554.77
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
360
380
400
420
440
460
480
500
520
540
560

c)


[M-2Br]·+
355.87
353.45
357.86
[M'-2Br]·+
386.17
384.35
388.24
[M'-BrCH3]·+
451.74
449.83
453.90
[M'-2OCH3]·+
[M-CH3]·+
[M]·+
515.69
513.74
517.73
502.28
500.0
504.5
487.25
485.55
489.18
519.73
[M']·+
545.75
543.75
547.72
541.70
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
360
380
400
420
440
460
480
500
520
540